

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : <b>C12Q 1/68, G01N 33/35, C12P 21/04, 21/06, C12N 5/00, C07K 1/00, 16/00, A01N 43/04, C07H 21/04</b>		A1	(11) International Publication Number: <b>WO 96/36730</b> (43) International Publication Date: 21 November 1996 (21.11.96)
(21) International Application Number: <b>PCT/US96/05386</b>		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 18 April 1996 (18.04.96)		Published <i>With international search report.</i>	
(30) Priority Data: <b>08/444,005 18 May 1995 (18.05.95) US</b>			
(71) Applicants: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02110-2804 (US). THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US).			
(72) Inventors: LEDER, Philip; 25 Aston Road, Chestnut Hill, MA 02167 (US). SEED, Brian; Apartment #5J, 9 Hawthorne Place, Boston, MA 02114 (US). STRANGER, Ben, Z.; 12 Alton Place, No. 6, Brookline, MA 02146 (US). LEE, Tae-Ho; LG Apartment #9-504, Doryong-dong 381-42, Yusung-gu, Daejeon (KR). KIM, Emily; 234 Reservoir Road, Chestnut Hill, MA 02167 (US).			
(74) Agent: CLARK, Paul, T.; Fish & Richardson, P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).			

(54) Title: CELL DEATH PROTEIN

(57) Abstract

Disclosed is a protein, designated RIP, which contains a death domain at its carboxy terminus and a kinase domain at its amino terminus. RIP interacts with the Fas/APO-1 intracellular domain and the TNFR1 intracellular domain. When expressed in transformed host cells, recombinant RIP promotes apoptosis. Also disclosed are DNA molecules encoding RIP, anti-RIP antibodies, and screening methods for discovering inhibitors of RIP-dependent apoptosis.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

- 1 -

CELL DEATH PROTEIN

Field of the Invention

This invention relates to recombinant DNA,  
5 proteins encoded by recombinant DNA, and screening  
methods for discovery of compounds with pharmacological  
activity.

Background of the Invention

Regulated (or "programmed") cell death is  
10 essential for the orderly development of metazoan  
organisms and is crucial to the proper functioning of the  
immune system in higher vertebrates (Wyllie et al., Int.  
Rev. Cytol. 68:251-306, 1980). The most common  
morphologic expression of programmed cell death is  
15 apoptosis, characterized by cell shrinkage, zeiosis, or  
blebbing, of the plasma membrane, and nuclear collapse  
and fragmentation of the nuclear chromatin, at  
intranucleosomal sites, due to activation of an  
endogenous nuclease (Clement et al., J. Exp. Med.  
20 180:557, 1994). Apoptosis is involved in morphological  
development, precise regulation of cell numbers, and as a  
defense mechanism to remove unwanted, and potentially  
dangerous cells, such as self-reactive lymphocytes, cells  
infected by viruses, and tumor cells. See, e.g.,  
25 Steller, Science 267:1445, 1995; Thompson, Science  
267:1456, 1995. Diseases associated with increased  
apoptosis include AIDS, neurodegenerative disorders  
(e.g., Alzheimer's disease, Parkinson's disease,  
amyotrophic lateral sclerosis, retinitis pigmentosa),  
30 aplastic anemia, ischemic injury (e.g., myocardial  
infarction, stroke, reperfusion injury), and toxin-  
induced (e.g., alcohol induced) liver disease.

An important mediator of immunologically relevant  
cell death is the Fas antigen/APO-1 (also known as CD95)  
35 ("Fas/APO-1"), originally identified as the target of

- 2 -

monoclonal antibodies that could kill multiple cell types (Trauth et al., Science 245:301, 1989; Yonehara et al., J. Exp. Med., 169:1747, 1989). Cloning of cDNA, followed by sequence analysis (Itoh et al., Cell 66:233, 1991; 5 Watanabe-Fukunaga et al., J. Immunol. 148:1274, 1992a; Oehm et al., J. Biol. Chem. 267:10709, 1992) showed Fas/APO-1 to be a member of a family of transmembrane receptors that includes the low affinity nerve growth factor ("NGF") receptor, the tumor necrosis factor 10 receptors ("TNFR1," "TNFR2"), and a variety of immune cell receptors including CD40, OX40, CD40, CD27, and 4-1BB (see, Smith et al., Cell 76:959, 1994). In addition to Fas/APO-1, several members of this family have been shown to regulate or induce cell death, e.g., p55 TNFR 15 (TNFR1) Tartaglia et al., Proc. Natl. Acad. Sci. 88:9292, 1991; Tartaglia et al., Cell 73:213, 1993b) and p75 TNFR (TNFR2) (Heller et al., Cell 70:47, 1992; Heller et al., Cell 73:216, 1993; Clement and Stamenkovic, J. Exp. Med. 180:557, 1994).

20 Disruption of Fas/APO-1 expression or function in lymphoproliferation ("lpr") mutant mice leads to a progressive lymphadenopathy and an autoimmune syndrome resembling human systemic lupus erythematosis (Watanabe-Fukunaga et al., Nature 356:314, 1992b). The residual 25 cytotoxic activity of T-cells derived from perforin deficient mice is also dependent on the presence of at least one wild type allele of the lpr locus (Kagi et al., Science 265:528, 1994; Kojima et al., Immunity 1:357, 1994; Lowin et al., Nature 370:650, 1994). Thus the 30 ability of Fas/APO-1 to induce cell death is important for the maintenance of at least two immunologic processes in vivo: peripheral tolerance to self (Singer and Abbas, Immunity 1:365, 1994; Crispe, Immunity 1:347, 1994), and calcium-independent T-cell cytotoxicity (Henkart, 35 Immunity 1:343, 1994).

- 3 -

The mechanism by which Fas/APO-1 induces cell death is unknown, but it requires multivalent cross-linking of the receptor (Dhein et al., J. Immunol. 149:3166, 1992) and is facilitated by concurrent inhibition of RNA or protein synthesis in some cell types. Other factors have been reported to modulate Fas/APO-1 activity (Klas et al., Int. Immunol. 5:625, 1993), and, under certain circumstances, Fas is capable of signaling activation rather than death (Alderson et al., J. Exp. Med. 178:2231, 1993). Anti-Fas antibodies and TNF are both capable of signaling cell death *in vitro* with similar kinetics (Yonehara et al., (supra); Itoh and Nagata, J. Biol. Chem. 268:10932, 1993), and among members of the NGF/TNF receptor family, Fas/APO-1 and TNFR1 share the most significant cytoplasmic homology.

Fas/APO-1 contains sequences required for the cell death response (Itoh and Nagata (supra) 1993). It has been proposed that a "death domain" contained in a region of similarity between Fas/APO-1 and TNFR1 is essential for the initiation of apoptosis by both molecules, perhaps through an interaction with other intracellular proteins (Tartaglia et al., Cell 74:845, 1993).

Summary of the Invention

We have discovered a protein, designated RIP (for Receptor Interacting Protein), which contains a death domain at its carboxy terminus and a kinase domain at its amino terminus. We have further discovered that RIP interacts with the Fas/APO-1 intracellular domain. Moreover, RIP overexpression in eukaryotic cells leads to apoptosis. The ability of RIP to associate with the Fas/APO-1 intracellular domain, and to promote apoptosis, indicates that it is an important element in the signal transduction machinery mediating programmed cell death.

Accordingly, the invention features an RIP, comprising a death domain in its carboxy terminal region

- 4 -

and a kinase domain in its amino terminal region, said RIP being capable of interacting with cellular factors such as the Fas/APO-1 intracellular domain, or the TNFR1 intracellular domain, and said RIP being capable of 5 promoting apoptosis in a eukaryotic cell. The invention also features RIP fragments, e.g., the RIP death domain, capable of interacting with one or more cellular factors and capable of promoting apoptosis.

The invention also features an isolated 10 recombinant DNA molecule encoding an RIP, or encoding a fragment thereof (e.g., the RIP death domain) and a host cell transformed with the recombinant DNA molecule. The transformed host cell is used to express recombinant RIP.

The invention also features anti-RIP antibodies, 15 which are useful as medical diagnostic reagents, as affinity chromatography reagents for isolation of RIP, and as analytical scale laboratory reagents for use in research on the physiology and cell biology of apoptosis.

The invention also features screening assays to 20 identify inhibitors of *in vivo* binding interactions mediated by the RIP death domain. Isolated RIP, recombinant RIP, or a binding domain thereof, is an essential component of the screening assays of this invention.

25 The invention also features screening assays to identify inducers of RIP expression and resulting apoptosis.

The invention also features methods of producing, for introduction into a patient undergoing gene therapy, 30 cells that can be induced to undergo apoptosis upon administration of an exogenous agent to the patient.

As used herein, the term "Act-Fas" means a transcriptional activator fusion protein expressed by a library isolate, wherein the activator is fused to a 35 Fas/APO-1 sequence.

- 5 -

As used herein, the term "Act-RIP" means a transcriptional activator fusion protein expressed by a library isolate, wherein the activator is fused to an RIP sequence.

- 5 As used herein, the term "apoptosis" means non-necrotic cell death that takes place in metazoan animal cells following activation of an intrinsic cell suicide program. Apoptosis is a normal process in the development and homeostasis of metazoan animals.
- 10 Apoptosis involves characteristic morphological and biochemical changes, including cell shrinkage, zeiosis, or blebbing, of the plasma membrane, and nuclear collapse and fragmentation of the nuclear chromatin, at intranucleosomal sites, due to activation of an
- 15 endogenous nuclease.

As used herein, the term "death domain" of RIP means the 98 C-terminal amino acid residues of RIP, which display sequence relatedness to an approximately 90-amino acid residue C-terminal region in Fas/APO-1 and TNFR1.

- 20 As used herein, the term "interacting" means specifically associating as a result of 3-dimensional structure; specific non-covalent binding.

- As used herein, the term "kinase domain" of RIP means the amino terminal region of RIP (i.e., from a few  
25 residues after the N-terminal methionine residue to approximately residue number 300), which has sequence relatedness to known tyrosine kinases and serine/threonine kinases.

- Other features and advantages of the invention  
30 will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### Brief Description of the Drawings

Fig. 1 is a graph summarizing quantitative  $\beta$ -galactosidase assay results. Three colonies from each  
35 Act-RIP/LexA-fusion protein pair were used to inoculate a

- 6 -

galactose-containing liquid culture. The  $\beta$ -galactosidase activity of lysates prepared from each culture was measured and normalized to the total protein concentration of the lysate. 5 units (nmoles/min/mg protein) represents the limit of detection of  $\beta$ -galactosidase activity in this system.

Fig. 2 is a set of genetic maps comparing RIP cDNAs. The inserts of clones isolated from a mouse thymus cDNA library are symbolized as lines above a bar diagram of the composite sequence, which depicts the regions encoding the kinase domain (hatched box), the death domain (black box), and a region of unknown function predicted to have high  $\alpha$ -helical content (unshaded). Also shown is the region of human RIP that was used as a probe to isolate murine cDNAs. The original human RIP isolate encompassed sequences encoding the death domain and about 100 residues upstream.

Fig. 3 is the deduced complete amino acid sequence of murine RIP and a deduced partial human RIP amino acid sequence.

(B) Deduced polypeptide sequence. The mouse sequence consists of a contiguous open reading frame proceeding from a translational initiation consensus. The human sequence predicted from a cDNA fragment is shown below the mouse; identical residues are indicated by a dash, and gaps indicated by a period. The conserved consensus sequences for casein kinase II (S-X-X-E) and cAMP- or cGMP-dependent protein kinase (R-X-X-S) are overlined.

Fig. 4 is the amino acid sequence of the RIP Death Domain. Sequence alignment of the RIP C-terminus with the death domains and carboxyl termini of Fas/APO-1 and TNFR1. Gaps are indicated by dashes. Consensus residues conserved in all six sequences are capitalized, whereas positions at which a charge-conserved residue is found in

- 7 -

1/6 sequences are shown in lower case. Noteworthy charge conservations are bolded. The regions denoted A and B represent two portions of the Fas/APO-1 cytoplasmic domain completely conserved between mouse and human.

5

Detailed Description

The present invention provides a novel recombinant protein, designated RIP, and DNA encoding the protein. RIP contains an amino-terminal kinase domain and a carboxy-terminal death domain, through which RIP 10 associates with the Fas/APO-1 intracellular domain, and to a lesser extent, the TNFR1 intracellular domain. RIP appears to be an apoptosis-inducing nonreceptor kinase. Accordingly, RIP appears to be an important element in the signal transduction pathway mediating programmed cell 15 death.

Various diseases, including AIDS, neurodegenerative disorders (e.g., Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, retinitis pigmentosa), aplastic anemia, ischemic injury 20 (e.g., myocardial infarction, stroke, reperfusion injury), and toxin-induced (e.g., alcohol induced) liver disease, involve abnormal increases in apoptosis. A specific inhibitor of an essential step in the biochemical machinery that mediates apoptosis is needed. 25 Such an inhibitor would be a drug candidate for therapeutic use against apoptosis-associated diseases. Because RIP interaction with (i.e., binding to) intracellular factors appears to be an essential step in triggering apoptosis, inhibitors of RIP binding to 30 intracellular apoptosis factors, e.g., Fas/APO-1 or TNFR1, are potential drug candidates. RIP (or a fragment thereof) is an essential component in any screening method for discovery of such RIP binding inhibitors.

RIP inhibitor screening methods, including cell-free methods and cellular methods, can be used in the 35

- 8 -

practice of this invention. Cellular screening methods within the scope of this invention can involve transient expression vectors or stable transformation. Various RIP inhibitor screening protocols can be designed, according 5 to well-known principles, by one of ordinary skill in the art, once RIP and RIP-encoding DNA are in hand, by virtue of the present invention.

Cell-free screening methods for inhibitors of RIP death domain-mediated binding involve the use of isolated 10 RIP and an RIP interaction partner, e.g., isolated Fas/APO-1 protein, TNFR1, or a polypeptide comprising the appropriate binding domain of one of these proteins. Soluble forms of RIP and RIP interaction partners can be utilized in cell free RIP inhibitor screening protocols. 15 Preferably, however, the cell-free RIP inhibitor screening protocol involves an RIP interaction partner inserted into a biological membrane. Membrane-inserted fusion protein forms of Fas/APO-1, can be produced, for example, using known sequences, according to the methods 20 of Clement et al. (*supra*).

Preferably, RIP inhibitor screening is carried out in a cellular system, using a reporter strain of cultured mammalian cells, transformed with one or more vectors encoding RIP, and other assay components, as necessary. 25 Preferably, an RIP-encoding sequence is cloned into a recombinant DNA vector, where it is expressed under the control of an inducible promoter, e.g., a heat shock promoter. See, e.g., Wurm et al., Proc. Natl. Acad. Sci. USA 83:5414, 1986. Following induction of RIP expression, Cell death is measured in experimental 30 treatments involving the presence of an inhibitor candidate, and in appropriate positive and negative controls. Various assays for cell death are known in the art, including the neutral red uptake method (Wallach, J. 35 Immunol. 132:2464, 1984), the crystal violet method (see,

- 9 -

e.g., Itoh et al., *supra*), or microscopic inspection of cells for visual signs of apoptosis.

Because overexpression of RIP can be used to induce apoptotic cell death, RIP is useful as a tool in gene therapy in at least two different ways: (1) to control the number of cells bearing a specific gene; and (2) to act as an anti-tumor agent in forms of cancer therapy that are dependent on the delivery of a lethal gene to neoplastic cells. In both applications, overexpression of RIP to cause apoptotic cell death is preferable to approaches employing death-inducing genes that result in in vivo generation of toxic agents or that interfere with cell cycle progression.

Cell ablation through RIP expression is advantageous because apoptotic death affects both mitotically active and mitotically quiescent cells. In contrast, chemotherapeutic agents and many gene therapy-based treatments for tumors require the target cell to be replicating in order for the treatment to be effective.

RIP genes used in gene therapy are preferably under the control of an exogenously regulatable promoter. An exogenously regulatable promoter is a promoter that can be induced by a specific set of environmental conditions, such as the increase in the concentration of a specific inducer. Examples of exogenously regulatable promoters and inducing conditions include: induction of a metallothionein promoter by zinc ions (Makarova et al., Nucleic Acids Res. 22:1504-1505, 1994), removal of tetracycline, thereby activating a synthetic promoter based on the action of a tetracycline repressor-VP16 chimera (Gossen et al., Proc. Natl. Acad. Sci. USA 89:5547-5551, 1992), addition of ecdysone (Christopherson et al., Proc. Natl. Acad. Sci. USA 89:6314-6318, 1992), or the synthetic progesterone antagonist mifepristone

- 10 -

(Wang et al., Proc. Natl. Acad. Sci. USA 91:8180-8184, 1994).

The RIP-encoding DNA of this invention enables one of ordinary skill in the art to produce anti-RIP antibodies. The RIP-encoding DNA is used to construct a vector encoding a fusion protein comprising an RIP moiety and an isolation-facilitating moiety, i.e., a moiety that can be readily isolated from contaminating proteins in an extract from a host cell used to express the fusion protein. A preferred isolation-facilitating moiety is maltose binding protein. DNA encoding maltose binding protein is commercially available. A binding reagent specific for the isolation-facilitating moiety is used for convenient and efficient isolation of the RIP fusion protein. For example, amylose chromatography is preferred for isolation of a fusion protein comprising maltose binding protein moiety. Following isolation, the RIP fusion protein is used to produce RIP-specific antibodies (polyclonal or monoclonal), according to standard methods, described, for example, by Harlow et al., (*Antibodies - A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988).

The anti-RIP antibodies of the invention have several uses. For example, they may be used as reagents for preparation of affinity chromatography media. Once the anti-RIP antibodies of this invention are in hand, preparation of RIP affinity chromatography media can be carried out according to conventional methods (see, e.g., Harlow et al., *supra*), using commercially available reagents. The RIP-specific affinity chromatography media can be used to isolate full-length RIP from natural sources or from host cells transformed with recombinant DNA encoding RIP.

The anti-RIP antibodies of the invention are also useful as analytical-scale laboratory reagents for

- 11 -

research on the physiology and cell biology of apoptosis. For example, immunohistochemical techniques, based on anti-RIP monoclonal antibodies are likely to be valuable tools for embryologists seeking ways to observe the rate 5 and/or distribution of apoptosis in the normal morphological development of metazoan animals.

The anti-RIP antibodies of the invention are also useful as diagnostic immunoassay reagents for measuring RIP levels in tissue samples from patients suspected of 10 having an apoptosis-related disease or abnormality.

Information on RIP levels in selected cells or tissues is a useful diagnostic or prognostic indicator in any situation where the rate of programmed cell death is important. The type of tissue sampled for the diagnostic 15 test will vary, depending on the signs and symptoms of the patient and the suspected disease or abnormality.

If the tissue sample is highly homogenous with respect to cell type, it may be preferable to carry out the RIP immunoassay on an extract from a homogenate.

20 Alternatively, it may be preferable to use an immunohistochemical assay involving anti-RIP antibodies. An immunohistochemical assay is preferable when the tissue sample is heterogenous with respect to cell type. An immunohistochemical assay will yield information on 25 the distribution of differing RIP levels in a cross section of tissue, or differing RIP levels in various types of blood cells in a blood sample.

Although inhibitors of RIP binding to intracellular apoptosis factors would be expected to have 30 therapeutic utility only for disease states involving increased apoptosis, information on the level of RIP in a tissue sample would have diagnostic/prognostic utility for any apoptosis-related disease, regardless of whether apoptosis was increased or decreased in that disease.

35 Examples of diseases associated with decreased apoptosis

- 12 -

include cancer (in particular, follicular lymphomas, carcinomas with p53 mutations, hormone-dependent tumors, e.g., breast cancer, prostate cancer, ovarian cancer), autoimmune disorders (e.g., systemic lupus erythematosus, 5 immune-mediated glomerulonephritis), viral infections, herpes viruses, poxviruses, adenoviruses).

The anti-RIP antibodies of the present invention can be used in various diagnostic immunoassay formats known in the art. Exemplary immunoassay formats are 10 competitive radioimmunoassay, ELISA, Western blot analysis and microcapillary devices comprising immobilized antibody. See, e.g., Dafforn et al., Clin. Chem. 36:1312, 1990; Li et al., Anal. Biochem. 166:276, 1987; Zuk et al., U.S. Patent No. 4,435,504; Zuk et al., 15 Clin. Chem. 31:1144, 1985; Tom et al., U.S. Patent No. 4,366,241; and Clark, PCT published application WO 93/03176.

The RIP-encoding DNA of this invention can be used as an *in situ* hybridization reagent to assess 20 transcription of RIP genes and observe RIP RNA processing, for diagnostic purposes or research purposes.

The RIP-encoding DNA of this invention can be obtained by screening a transcriptional activator fusion library from the species of interest, by means of a 25 protein interaction system, as was done in the original work on this invention, and which is described in detail, below. Preferably, however, RIP-encoding DNA is obtained through a more direct approach, using nucleotide sequences or amino acid sequence information provided 30 herein.

A general method for cloning a full length human RIP cDNA involves screening a human cDNA library (randomly primed from any tissue) with a probe derived from the 5' region of the murine RIP coding sequence,

- 13 -

using a low stringency procedure similar to that described under the "cDNA Cloning" heading, below.

A wide variety of host/expression vector combinations can be employed for expressing RIP-encoding DNA of this invention. The expression of RIP-encoding DNA in a cellular screening assay is preferably in a eukaryotic cell, under the control of eukaryotic expression control sequences. More preferably, the eukaryotic cell is a cultured mammalian cell. If the expression of recombinant RIP-encoding DNA is merely for the production of isolated recombinant RIP, however, a prokaryotic host/expression vector system or a eukaryotic host/expression system can be used.

In the studies leading to the present invention, a yeast two-hybrid screen of a human T-cell cDNA library identified two proteins capable of interacting with the intracellular domain of Fas/APO-1: Fas/APO-1 itself and a novel protein, RIP, containing a death domain and a kinase domain. Neither protein interacts with a humanized variant of the murine *lpr<sup>cg</sup>* allele, which confers much the same phenotype on affected mice as does the original *lpr* allele (Matsuzawa et al., 1990). The finding that the intracellular domain of the *lpr<sup>cg</sup>* variant is incapable of interacting with the wild type Fas/APO-1 cytoplasmic domain in yeast suggests that oligomerization of Fas/APO-1 mediated by cytoplasmic sequences may be a prerequisite for activity. However it has not been established whether oligomerization of Fas/APO-1 is necessary for interaction with RIP, or whether the same subdomain exploited for oligomerization is also required for heteromeric association with RIP. The discovery of the Fas-Fas interaction in a library screen is consistent with a recent demonstration that TNFR1 and Fas/APO-1 are capable of both self- and cross-association in yeast (Boldin et al., J. Bi l. Chem. 35

- 14 -

270:387, 1995). The death domain probably mediates such interactions, because work on this invention indicates that RIP can also associate with the intracellular domain of TNFR1 (as well as the intracellular domain of Fas/APO-1).

RIP mRNA is expressed at low levels in all tissues. A pattern of widespread expression has also been observed for Fas/APO-1. In contrast to RIP, however, Fas/APO-1 mRNA is found at higher levels in thymus, liver, lung, and heart. Like Fas/APO-1 mRNA, RIP mRNA is induced in splenocytes after activation with ConA. Although a requirement for RIP in Fas-mediated killing has not been established, coordinate induction of Fas/APO-1 and RIP may contribute to increased susceptibility of T-cells to Fas-mediated cell death following activation.

The N-terminus of RIP contains a kinase domain that has features of both Ser/Thr and Tyr kinases. In the two interacting loops that appear to control hydroxyamino acid recognition (Hubbard et al., Nature 372:746, 1994; Taylor et al., Structure 2:345, 1994), RIP closely resembles a Ser/Thr kinase. In particular, it lacks the Ala-X-Arg or Arg-X-Ala motif in the catalytic loop (subdomain VI) and the Pro-X-X-Trip motif in the P+1 loop (subdomain VIII), both of which are found in all Tyr kinases (see, e.g., Hanks et al., in Meth. Enzymol. 200, Academic Press, at pp. 38-62, 1991). Other support for the possibility that RIP has either mixed catalytic specificity or Tyr kinase-like regulation with Ser/Thr specificity, comes from the finding that, among known sequences, RIP shows the greatest overall primary sequence similarity to murine lck, a tyrosine kinase of the src family. The relatedness to tyrosine kinases is especially apparent among the framework residues outside the active site which make up the C-terminal, alpha

- 15 -

- helic-rich substrate-binding lobe. For example, RIP has a tryptophan at position 269 which is present in all Tyr kinases analyzed by Hanks et al. (Science 241:42, 1988), but absent from all Ser/Thr kinases examined, except Mos.
- 5 The presence of structural motifs from both Tyr and Ser/Thr kinases has also been noted for the soybean kinase GmPK6 (Feng et al., Biochim. Biophys Acta 1172:200, 1993), which shows high global similarity to RIP. Although the exact role of protein kinases in
- 10 Fas/APO-1 mediated apoptosis is unknown, it appears that the participation of at least one protein tyrosine kinase is required.

In work on this invention, overexpression of RIP resulted in the induction of a cell death program 15 morphologically indistinguishable from apoptosis. Deletion of the C-terminal region of RIP spanning the segment of death domain homology eliminated the apoptotic response, but deletion of the kinase domain did not entirely quench activity.

- 20 Although the evidence that RIP binds to Fas/APO-1 in yeast favors a model in which RIP acts directly downstream of Fas/APO-1 in a death pathway, it is also possible that RIP has other actions. For example, the elevation of RIP mRNA in activated T-cells 25 suggests that RIP may be involved in the apoptosis provoked by growth factor deprivation.

#### Experimental Information

##### RIP cDNAs

- A cDNA library screen for proteins that 30 interact with the intracellular domain of Fas/APO-1 was conducted by using a variation of the yeast interaction system described by Gyuris et al. (Cell 75:791, 1993). The assay system involved a yeast reporter strain containing two separate reporter genes, *S. cerevisiae*

- 16 -

leu-2 and *E. coli*  $\beta$ -galactosidase coding sequences under the control of a synthetic promoter which contain a Lex-A binding site, and which required a trans-acting transcriptional activator in order for transcription to occur. A cDNA segment comprising most of the cytoplasmic domain of human Fas/APO-1 (residues 192 to 329 of the Fas precursor) was fused to the 3' end of the coding region for the bacterial repressor LexA, in a yeast expression plasmid, which was used to transform the reporter strain. This Fas/LexA construct encoded a transcriptionally inert fusion protein ("the bait"), capable of specific binding at the LexA binding site in the reporter gene promoters, by virtue of the LexA moiety. The bait-expressing reporter strain was transformed with a transcriptional activator fusion protein library prepared from mRNA isolated from the Jurkat (human T-cell leukemia) cell line, which is known to undergo apoptosis when subjected to treatment with anti-Fas antibody. The transcriptional activator moiety of the library-encoded fusion proteins lacked DNA binding activity. Any transcriptional activator fusion protein with the ability to bind to the Fas/APO-1 intracellular domain, however, would bring the transcriptional activator moiety into proximity with the reporter gene promoter, thereby resulting in transcription of the reporter genes.

Transformants were plated on selective (leucine deficient) plates containing galactose, which induces the GAL1 promoter that directs transcription of the library insert. Leucine prototrophs were transferred to plates containing X-gal and galactose, and colonies giving a dark blue color reaction were recovered and analyzed further.

To test the specificity of the interaction between the candidate interaction partners and Fas/APO-1, the library plasmids were reintroduced into a second

- 17 -

strain harboring a LexA-Fas fusion gene (LexA-lpr<sup>cg</sup>) in which the Fas portion had been mutated by substitution of asparagine for valine at position 254 of the Fas/APO-1 precursor sequence. This mutation was expected to  
5 exhibit a molecular phenotype similar to that of the murine lpr<sup>cg</sup> allele, which is formed by substitution of asparagine for isoleucine at the homologous position. None of the candidate plasmids showing evidence of strong interaction with LexA-Fas were capable of interacting  
10 with LexA-lpr<sup>cg</sup> (Figure 1A; data not shown).

Restriction site and sequence analysis of the cDNA inserts of the candidate clones showed all to be incomplete cDNAs falling into only two classes. One of the inserts encoded the C-terminal residues 222 to 335 of  
15 the intracellular domain of Fas/APO-1 itself. The other encoded a protein, designated RIP, which had no overt relationship to previously described polypeptides.

The specificity of the interaction partners was further tested by using the library plasmids to transform  
20 yeast harboring expression plasmids encoding LexA fusions with intracellular domains of various cell surface receptors. Although no interaction was detected in most cases, weak interaction was detected following introduction of the yeast plasmid encoding Act-RIP into  
25 strains harboring LexA-TNFR1 intracellular domain. By contrast, no activity was seen when Act-RIP was introduced into strains harboring LexA-TNFR2 intracellular domain. Yeast transformed with Act-Fas displayed promoter activity in strains harboring LexA-Fas  
30 intracellular domain, but showed no activity in strains harboring any other LexA-intracellular domain.

To measure this effect more precisely,  $\beta$ -galactosidase assays were performed on lysates of yeast harboring various pairs of LexA-intracellular domain and  
35 Act-RIP. Lysates from yeast bearing LexA-Fas and Act-RIP

- 18 -

contained about 30 to 40 fold more  $\beta$ -galactosidase activity than strains bearing Act-RIP and either LexA-lpr<sup>cg</sup> or LexA-TNFR2 (Fig. 1). Lysates prepared from yeast harboring LexA-TNFR1 and Act-RIP expressed  $\beta$ -galactosidase activity at about 10% of the level seen in lysates prepared from yeast bearing LexA-Fas and Act-RIP (Fig. 1).

One explanation for the failure to detect an interaction between Act-RIP and LexA-lpr<sup>cg</sup> or LexA-TNFR2 could have been that the LexA fusion proteins were poorly expressed. To address this possibility, a portion of each lysate used to measure enzyme activity was subjected to gel electrophoresis and blot transfer, followed by detection with anti-LexA antiserum. LexA fusion proteins of the appropriate size were detected in each of the lysates and both the LexA-lpr<sup>cg</sup> and LexA-TNFR2 fusion proteins were found to be more abundantly expressed than LexA-Fas or LexA-TNFR1 (data not shown), making it unlikely that failure to detect interaction *in vivo* could be attributed to degradation or inadequate synthesis of the LexA chimeras.

#### Cloning and Structure of Murine RIP

Because the RIP cDNA insert identified in the two hybrid screen did not encode an open reading frame bearing a consensus translational initiation sequence, additional human cDNA libraries were screened by hybridization for a full length clone. Initially, none were identified, however, and several of the resulting isolates appeared to terminate at approximately the same 5' terminus, suggesting that secondary structure in the mRNA might have prevented extension of the cDNA by reverse transcriptase. (Subsequently, overlapping cDNA clones representing the entire human RIP coding sequence were obtained.) Although the largest clone spanned approximately 1 kb of sequence, preliminary RNA blot

- 19 -

hybridizations revealed a transcript of approximately 4.2 kb expressed in cell lines of diverse provenance, including tumors of lymphoid, hepatic, renal, neuronal, cervical, intestinal, muscular and skeletal origin.

5 To isolate a full length murine RIP clone, the human RIP coding sequence was used to probe a mouse thymus cDNA library. Four distinct overlapping clones were identified, ranging in size from 1 kb to 2.4 kb, as shown in Fig. 2. Restriction analysis and sequencing  
10 revealed that one of these clones, III2, extended further 5' than the others. The 2.3 kb insert of III2 contains a 1968 nucleotide open reading frame beginning with a translational initiation consensus sequence and predicting a polypeptide of 656 amino acids (SEQ ID  
15 NO:15) with  $M_r$  of 74,000 (Fig. 3). The nucleotide sequence of a full-length murine RIP cDNA is (SEQ ID NO:14).

The amino terminal region of RIP bears an extended homology to protein kinases that begins a few  
20 residues after the presumptive initiating methionine and extends to the vicinity of residue 300. Quantitative sequence comparisons based on a word match algorithm (Altshul et al., J. Mol. Biol. 215:403, 1990) predict that this domain is most similar overall to the tyrosine  
25 subclass of protein kinases, with the highest relatedness seen to the mouse *Ick* gene product (Marth et al., Cell 43:393, 1985). However in the key subdomains that discriminate most closely between tyrosine and serine/threonine substrate specificity, the HKDLKPen  
30 motif of the catalytic loop (kinase subdomain VI) and the GTLYYMAPE sequence of the P+1 loop (kinase subdomain VIII), RIP appears to match the serine/threonine family consensus (Hanks et al., in Methods in Enzymology vol. 200, (Hunter et al., eds.), Academic Press, pp. 38-62,  
35 1988; Taylor et al.; supra).

- 20 -

The sequence predicted by one of the human cDNA fragments consists of 375 amino acids corresponding to the region just C-terminal to the kinase domain of murine RIP, and shares 67% sequence identity with the murine sequence over this length. Within this portion, the first 270 amino acids following the kinase domain have no striking homology to other proteins, although a small subdomain within this portion is highly conserved between mouse and human proteins (residues 391 to 427 of the murine sequence) and has a relatively high representation of Arg (R), Gln (Q) and Glu (E) (18/37 residues in both sequences). The sequence of this region is similar to portions of the trichohyalin family of hair structural proteins, which contain RQE-rich repeats that form highly stabilized alpha helices (Lee et al., J. Biol. Chem. 268:12164, 1993).

Complete Human RIP Coding Sequence

Subsequent to the cloning and sequencing of a full-length murine RIP cDNA (SEQ ID NO:14), a complete human RIP coding sequence was obtained from sequence analysis of multiple overlapping partial cDNAs. A complete human RIP coding sequence is (SEQ ID NO:16). The deduced amino acid sequence of a full-length human RIP is (SEQ ID NO:17).

25 RIP C-terminus Death Domain Homology

The remaining 98 C-terminal amino acids share 87% sequence identity between mouse and human RIP, suggesting they subserve some regulatory function (Fig. 3). Comparable domains of approximately 90 residues close to the C-termini of Fas/APO-1 and TNFR1 have been shown to play a role in the transduction of apoptotic signals to receptive cells, and have been termed "death domains" for this reason (Tartaglia et al., Cell 74:845, 1993). Pairwise comparisons of the death domain

- 21 -

sequences aligned in Fig. 4 showed the highest relatedness between human RIP and human TNFR1 (59% similarity and 30% identity), which are significantly more similar than human Fas/APO-1 and human TNFR1 (42% similarity and 23% identity). The interspecies conservation of the RIP death domain (85% identity between mouse and human) exceeds that of the TNFR1 (68% identity) and Fas/APO-1 (49% identity) death domains.

Constitutive and Inducible Expression of RIP mRNA

10 Preliminary RNA blot hybridization experiments demonstrated the existence of an RNA species of approximately 3.8 kb in a variety of cell lines. To more precisely assess mRNA abundance in tissues a quantitative ribonuclease protection assay was employed. Use of a  
15 labeled antisense RNA probe corresponding to the 3' terminus of the cDNA gave rise to a ribonuclease resistant species of the expected size in all adult tissues tested. An in vitro labeled RNA antisense to the mRNA for the ribosomal large subunit protein L32 was used  
20 as an internal standard to allow normalization to the amount of RNA loaded in each lane. Analysis of the protected RNA showed that RIP mRNA levels varied by less than 2-3 fold between most tissues (not shown); lung showed the highest expression, whereas tongue showed the  
25 least.

The possibility that RIP mRNA might be regulated as a consequence of activation in T-cells was also explored. Dissociated murine splenocytes were stimulated *in vitro* with the lectin concanavalin A and  
30 total RNA prepared at various times following addition of lectin was analyzed for the presence of RIP sequences by RNA blot analysis. Little or no RIP RNA could be detected in unstimulated splenocytes, but a single 3.6-  
3.8 kb species appeared in unfractionated splenocytes  
35 that had been exposed to lectin for 2 h or longer. Since

- 22 -

RIP mRNA is detectable by ribonuclease protection in the spleen as a whole, the inability to detect RIP mRNA in splenocytes treated with ConA for less than 2 h is probably due to the lower sensitivity of RNA blot analysis, although this discrepancy could also have resulted from RIP expression exclusively in the fibrous tissue of the spleen.

Immunodetection of RIP Produced In Vivo

To examine the distribution of RIP protein in vivo, a rabbit antiserum was prepared against a fusion protein consisting of the 250 C-terminal residues of murine RIP fused to *E. coli* maltose binding protein. The antiserum specificity was validated by immunoprecipitation of RIP synthesized in vitro.

Following in vitro transcription and translation of the RIP open reading frame, a single labeled product of approximately 74 kD was observed which could be specifically immunoprecipitated with the rabbit antiserum, but not with serum from unimmunized animals.

The specificity of the antiserum for RIP was also documented by its inability to immunoprecipitate an irrelevant protein (*P. pyralis* luciferase) similarly translated in vitro. Immunoprecipitation of a lysate of metabolically labeled NIH3T3 cells with the rabbit antiserum revealed the presence of a single protein species with the same molecular mass as that revealed by in vitro translation of RIP.

Detection of RIP in Transiently Transfected BHK Cells

To determine whether RIP protein could have a direct effect on cell viability, BHK cells grown on coverslips were transiently transfected with a epitope-tagged version of RIP (RIPmyc). Transfected cells were reacted with anti-RIP antiserum or an anti-myc monoclonal antibody. Weak expression was detected with both antibodies. The pattern of immunoreactivity was

- 23 -

heterogeneous, with both diffuse cytoplasmic as well as punctate perinuclear patterns observed. DNA staining with Hoechst 33258 showed that many of the RIP-expressing cells had apoptotic nuclei, a feature not seen when 5 vector or Fas control expression plasmids were used. However, a number of RIP-expressing cells could be found that had normal-appearing nuclei; conversely, apoptotic cells having no detectable RIP staining were also seen.

The concordance of cell death and RIP 10 expression suggested the ability to detect RIPmyc protein might be compromised by the death of the cells in which it was being expressed. To test this, and the possible role of individual RIP domains in apoptosis, two additional epitope-tagged constructs were prepared: one 15 lacking C-terminal sequences, including the death domain (RIPmycΔdeath), and one lacking ~ 200 amino acids in the kinase domain (RIPmycΔkinase). Both deletion mutants showed greater immunoreactivity with anti-myc antibodies than the full length construct. Only RIPmycΔkinase was 20 detected by the anti-RIP antiserum, as expected from the deletion of its epitope from RIPmycΔdeath.

RIP Overexpression Leads to Cell Death

To determine whether RIP was inducing cell death, we marked the transfected cells by co-transfection 25 with β-galactosidase. Cells were transfected with pairs of expression plasmids encoding RIPmyc and β-galactosidase (Price et al., 1987) at a 1:3 ratio of β-gal plasmid to RIP plasmid. After histochemical detection of β-galactosidase activity, cells transfected 30 with β-gal and RIPmyc expression plasmids were found to contain a large proportion of intensely staining, shrunken blue cells that exhibited membrane blebbing and loss of adherence. By contrast, transfection with β-gal plasmid, either alone or in combination with RIPmycΔdeath 35 plasmid, had no adverse effect upon nuclear morphology

- 24 -

and resulted in a predominantly cytoplasmic  $\beta$ -galactosidase staining pattern. Co-transfection of  $\beta$ -gal plasmid with RIPmyc $\Delta$ kinase likewise gave cytoplasmic staining of healthy-appearing cells, although a number of 5 shrunken, blebbled cells were also seen.

To quantify these results, cells from three independent transfections were examined and the morphologically apoptotic blue cells enumerated as a fraction of total blue cells. Over 57 percent of blue 10 cells arising from co-transfection of RIPmyc and the  $\beta$ -gal plasmid showed morphological changes consistent with apoptosis, whereas only 1-2 percent of blue cells that had been transfected with  $\beta$ -gal plasmid alone or in combination with RIPmyc $\Delta$ death exhibited such a phenotype. 15 However a consistent low frequency of apoptotic changes was seen in co-transfections involving RIPmyc $\Delta$ kinase, with almost 11 percent of the blue cells appearing to have undergone cell death. The fraction of cells showing morphological changes was positively correlated with the 20 ratio of RIPmyc or RIPmyc $\Delta$ kinase plasmid to  $\beta$ -gal plasmid, so that increasing RIP: $\beta$ -gal plasmid ratios gave higher percentages of dead blue cells (data not shown).

#### Plasmid Construction

The yeast interaction system was modified from 25 that described by Gyuris et al. (supra) by engineering the LexA expression plasmid to remove an internal MluI site and to insert MluI and NotI sites downstream from the DNA portion encoding the C-terminus of the gene. The resulting distal polylinker has the site sequence MluI-PmeI-NotI-EcoRI in the frame in which the MluI site 30 encodes Thr and Arg (frame 1).

The DNA encoding receptor cytoplasmic tails were amplified by PCR from cDNA libraries and cloned as 35 MluI-NotI or BssHII-NotI fragments using the following oligonucleotide primers: Fas: 5'-CGCGGGACGC GTAAGGAAGT

- 25 -

ACAGAAAACA TGC-3' (SEQ ID NO:1) and 5'-CGCGGGGCGG  
CCGCTCTAGA CCAAGCTTTG GATTTC-3' (SEQ ID NO:2); TNFR1: 5'  
CGCGGGGCGC GCTACCAACG GTGGAAGTCC AAG-3' (SEQ ID NO:3) and  
5' CGCGGGGCGG CCGCTGCCCG CAGGGGCGCA GCCTCA-3' (SEQ ID  
5 NO:4); and, TNFR2: 5'-CGCGGGACGC GTAAGAAGCC CTTGTGCCTG  
CAG-3' (SEQ ID NO:5) and 5'-CGCGGGGCGG CCGCTTTAAC  
TGGGCTTCAT CCC AGC-3' (SEQ ID NO:6).

The Fas/APO-1 cytoplasmic domain used in the library screen diverges at the Glu located five residues 10 prior to the C-terminus, and continues an additional 25 residues through vector sequences to the C-terminus. In all subsequent analyses, these residues were found not to contribute detectably to either Fas-Fas or Fas-RIP interaction.

15 A mutant Fas bait protein analogous to the *lpr<sup>cg</sup>* point mutation was made by mutating the valine at position 254 of human Fas to asparagine, using the following oligonucleotides in a recombinant PCR reaction:  
5'-CGAAAGAATG GTAAACAATGAA GCC-3' (SEQ ID NO: 7), and  
20 5'-GGCTTCATTG TTACCATTCTT TCG-3' (SEQ ID NO: 8).

In the resulting construct, residues 330 and 331 were also converted from Glu and Ile to Gly and Asn, respectively.

A myc-tagged version of RIP (RIPmyc) was made  
25 by digesting RIP clone III2 with TfiI and ligating a HindIII/TfiI adaptor to the 5' end and TfiI/NotI adaptor containing the myc epitope and a stop codon to the 3' end. The sense (#145) and antisense (#146) oligonucleotides comprising the 3' adaptor were as  
30 follows:

#145: 5'-ATTCGTGCCA GCCAGAGCGG CATGGAGCAG AAGCTCATCT  
CAGAAGAAGA CCTCGCGTAA GC-3' (SEQ ID NO:9), and

#146: 5'-GGCCGCTTAC GCGAGGTCTT CTTCTGAGAT GAGCTTCTGC  
TCCATGCCGC TCTGGCTGGC ACG-3' (SEQ ID NO:10).

- 26 -

The resulting insert was cloned into the HindIII and NotI sites of pcDNA I (Invitrogen).

To make RIPmycΔkinase, a PCR reaction was performed using a 5' RIP primer (#160: 5'-CCCAAGCTTG 5 TTGGAGATTC TGAGCAATC-3' (SEQ ID NO:11)) and an internal kinase domain primer (#161: 5'-CCCGATCTGC AGGTCAATGTA AGTCGCACATGCC-3 (SEQ ID NO:12)). The resulting product was cloned into the HindIII and PstI sites of RIPmyc resulting in the deletion of RIP residues 132 to 323.

10 RIPmycΔdeath was made by PCR, using a T7 primer and a myc-tag-containing primer (#151: 5'-CCCCTCGAGT TAGAGGTCTT CTTCTGAGAT GAGCTTTGC TCTTTCTTTA AACTTGCCAC-3' (SEQ ID NO: 13)). The amplified RIPmycΔdeath sequence, lacking amino acids 309 to 656, was subcloned as a 15 HindIII/XhoI fragment into a pcDNA I. Myc tags were located at the C-terminus of all proteins, and thus detection of the myc epitope requires translation of the entire cloned sequence. The BAG retrovirus vector encoding β-galacto-sidase has been described (Price et 20 al., Proc. Natl. Acad. Sci. USA 84:156, 1987).

Yeast Strains and Library Screen

Yeast transformation with library DNA was performed according to the method of Schiestl and Gietz (Curr. Genet. 16:339, 1989). Recipient cells, 25 EGY48/pRB1840 (Gyuris et al, supra) bearing LexA-fusion protein plasmids, were grown overnight in YPAD medium to a density of approximately  $10^7$  cells/ml, then diluted in 100 ml of warmed YPAD to a density of  $2 \times 10^6$  cells/ml and regrown to  $10^7$  cells/ml. The cells were harvested and 30 washed in water, resuspended in 1 ml water, transferred to a sterile microcentrifuge tube and pelleted. The pellet was resuspended in 0.5 ml of 10mM Tris HCl (pH 7.5), 1 mM EDTA, 0.1 M Li acetate, (pH adjusted to 7.5 with acetic acid and passed through 0.2 micron filter). 35 50 μl of the resulting suspension was mixed with 1 μg of

- 27 -

transforming DNA and 50 µg of single stranded salmon sperm DNA, after which 0.3 ml of a solution of 40% polyethylene glycol-4000 in Tris/EDTA/LiOAc was added and mixed thoroughly, followed by incubation at 30°C with 5 agitation for 30 min. After a heat pulse at 42°C for 15 min, the cells were pelleted in a microcentrifuge and the pellets resuspended in 1 ml of Tris/EDTA, diluted and plated on selective medium. Library screening and recovery of plasmids was performed as described by Gyuris 10 et al. (supra).

For assessing the interaction of RIP and Fas with other bait proteins, cells of the yeast strain EGY48/pSH18-34, containing eight copies of the LexA operator-lacZ reporter, were transformed with each of the 15 four bait constructs and selected on Ura<sup>-</sup>His<sup>-</sup> glucose plates. These bait strains were subsequently used for transformation of the RIP or Fas library plasmids and plated on Ura<sup>-</sup>His<sup>-</sup>Trip<sup>-</sup>Xgal plates containing either 2% glucose or 2% galactose/1% raffinose. The LexA-TNFR2 20 bait strain gave a weak blue color reaction when grown on galactose, indicating spontaneous transcriptional activation by the fusion protein.

β-Galactosidase Assays and Immunoblotting

Assays of crude extracts were carried out as 25 described (Rose et al., 1990). In brief, cells bearing the appropriate bait and interaction plasmid were grown to saturation overnight at 30°C in minimal Ura<sup>-</sup>His<sup>-</sup>Trip<sup>-</sup> medium with 2% glucose. The next day, cells were diluted 1:50 into medium containing 2% galactose and 2% raffinose 30 and allowed to grow overnight. Cells were spun and resuspended in breaking buffer (100 mM Tris (pH 8), 20% glycerol (v/v), 1 mM DTT). Half of each suspension was transferred to a separate tube, and PMSF was added to a final concentration of 10 mM. Cells were lysed by 35 vortexing with acid-washed beads, and the resulting

- 28 -

lysate was cleared by centrifugation. Enzyme activity was measured by incubating in Z buffer (Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, 1972) with 0.67 mg/ml o-nitrophenyl- $\beta$ -D-5 galactoside substrate. Reactions were stopped with 0.5 ml 1M Na<sub>2</sub>CO<sub>3</sub> when an appropriate level of color had developed. The protein concentrations of the lysates were determined by Bradford assay. Units of  $\beta$ -galactosidase were calculated using the following 10 equation: specific activity  
$$(nmoles/min/mg) = (OD_{420} \cdot 378) / (\text{time(min)} \cdot \text{vol extract(ml)} \cdot \text{concentration (mg/ml)}).$$

cDNA Cloning

Additional clones overlapping the primary RIP 15 isolate were sought in two libraries -- an expression library prepared in the CDM8 plasmid vector using mRNA isolated from the human cytolytic T-cell line WH3, and from a commercially available human leukocyte library in lambda phage (Clontech; HL1169a). Both libraries were 20 screened by filter replica hybridization, using radiolabeled probes derived from the insert isolated by interaction screening, as well as from subsequent inserts identified by hybridization.

To isolate murine RIP cDNA clones, 1.2 kb of 25 human RIP sequence was subcloned into two halves and each fragment used to probe genomic DNA blots containing mouse and human DNA to optimize conditions for cross-species hybridization. A commercially available oligo(dT)-primed mouse thymus cDNA library (Stratagene) from a (C57B1/6 X 30 CBA)F<sub>1</sub> mouse was plated out, and 10<sup>6</sup> plaques were screened using each of the human fragments on duplicate Genescreen filters (DuPont). Probes were synthesized using the random hexamer method Feinberg et al., Anal. Biochem. 132:6, 1983). Hybridization conditions were 5X SSPE, 10X 35 Denhardts, 2% SDS, 0.1 mg/ml herring sperm DNA at 55°C

- 29 -

overnight. Filters were washed in 2X SSC, 0.1% SDS at 55°C with several changes over 1 h. Plaque purified phage were isolated with three rounds of screening (Sambrook et al.; supra), and in vivo excision carried 5 out using Exassist phage and SOLR™ recipient cells (Stratagene). Seven independent clones were isolated, and they fell into the four classes shown in Fig. 2. The coding sequence of RIP was obtained as a composite from the cDNA clones sequenced on both strands using Sequenase 10 T7 polymerase (U.S. Biochemical). Several nucleotide polymorphisms were detected between the multiple clones, only one of which resulted in an amino acid difference: a Thr to Ile at position 473. Sequence comparisons were done with Genetics Computer Group, Inc. or MegAlign 15 (DNASTar, Inc.) software using default parameters.

RNAse Protections and RNA Blot Hybridization

Tissue RNA samples were prepared from wild type FVB mice (Taconic) by guanidinium thiocyanate lysis and centrifugation through a CsCl cushion (Chirgwin et al., 20 Biochemistry 18:5294, 1979). An antisense probe for RIP made from cDNA clone III1, linearized with SpeI, was synthesized using T7 polymerase with an *in vitro* transcription kit (Stratagene), with the addition of 20 μM cold rUTP and 100 μCi  $^{32}$ P-UTP (New England Nuclear). 25 The ribosomal L32 probe was synthesized from an XbaI-linearized template at one tenth the specific activity of the RIP probe. The use of L32 as an internal control for RNA loading has been described elsewhere (Shen and Leder, Proc. Natl. Acad. Sci. USA, 89:8240, 1992). As expected, 30 the *in vitro* transcription products were slightly larger than the protected fragments of 525 and 279 nucleotides, respectively. After removal of the DNA template with DNaseI, probes were purified using an ultrafree-MC 30,000 retention filter (Millipore). A sample ( $2 \times 10^5$  cpm) of 35 the RIP probe and a sample of L32 ( $2 \times 10^4$  cpm) were mixed

- 30 -

with 10 µg of RNA from each tissue or 50 µg of tRNA and allowed to hybridize in PIPES buffer with 50% formamide overnight at 50°C. Following this incubation, unhybridized RNA was digested with RNase A (40 µg/ml) and 5 RNase T<sub>1</sub> (1.3 µg/ml) at 37°C for 15 min. Samples were extracted with phenol/chloroform once, ethanol precipitated, washed, and boiled for 3 min before loading on a 6% polyacrylamide gel. After electrophoresis, gels were dried and exposed and radioactivity was quantitated 10 with the aid of a Molecular Dynamics Phosphorimager.

For measuring RIP in activated T-cells, spleens were removed from adult animals and dissociated using a stainless steel mesh. Cells (~10<sup>7</sup> per timepoint) were exposed to ConA (10 µg/ml) for 0-4.5 h, and RNA prepared 15 as described above. Approximately 8 µg of total RNA from each sample was loaded on a 0.9% agarose/4% formaldehyde gel, electrophoresed in 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, and transferred to nylon filters (GeneScreen, DuPont) by capillary transfer (Sambrook et al., supra). 20 Sizes were estimated using a molecular weight standard (Gibco BRL). The blot was hybridized with either an RIP probe corresponding to the C-terminal half of the protein (cDNA III1) or with a probe detecting ribosomal 28S RNA. Hybridization was allowed to proceed overnight in 40% 25 formamide, 4X SSC, 10% dextran sulfate, 7 mM Tris (pH 7.6), and 20 µg/ml salmon sperm DNA at 42°C. The blots were then washed at 50°C in 1X SSC, 0.1% SDS (RIP probe) or 60°C in 0.1X SSC, 0.1% SDS (28S probe), and exposed to film with intensifying screens.

30 RIP Antisera and Immunoprecipitations

Rabbit polyclonal antisera recognizing RIP were prepared by Pocono Rabbit Farm & Laboratory, Inc. (Canadensis, PA) using a fusion protein containing the C-terminal 250 amino acids of mouse RIP fused to maltose-35 binding protein (MBP; New England Biolabs). MBP-RIP was

- 31 -

purified by amylose chromatography and acrylamide gel elution. For immunoprecipitations, RIP was first transcribed in vitro using 15 µg full-length RIP template in a 300 µl reaction containing 3.3 mM each ATP, GTP, 5 CTP, and UTP, 280 units RNase inhibitor, 400 units Sp6 RNA polymerase and 1X Sp6 buffer (Boehringer Mannheim Biochemicals) at 37°C for 2 h. Translations were carried out using a reticulocyte lysate kit containing a luciferase positive control (Oromega) using approximately 10 4 µCi <sup>35</sup>S-Met per reaction. Reaction products were diluted to 1 ml in a modified RIPA buffer (0.5% NP40, 0.5% sodium deoxycholate, 0.025% SDS, 50 mM Tris, 30 mM sodium pyrophosphate, 50 mM NaF, 100 µM sodium orthovanadate [pH7.6]) containing protease inhibitors and 15 precleared with 50 µl normal rabbit serum and 100 µl protein A sepharose (1:1). Reactions were split in half, 5 µl preimmune or immune serum was added to each, and samples were allowed to rotate for 1 h at 4°C. Complexes were precipitated with 40 µl protein A sepharose (1:1), 20 washed 3 times with modified RIPA buffer, and resolved by SDS-PAGE.

For immunoprecipitation from metabolically labeled cells, a 150 mm plate of subconfluent NIH3T3 cells were incubated overnight in methionine-deficient 25 DMEM (Gibco/BRL, catalog #11970-019) supplemented with <sup>35</sup>S-methionine (~100 µCi/ml), 5% dialyzed fetal calf serum, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml). Cells were lysed for 15 min on ice in lysis buffer (250 mM NaCl, 50 mM HEPES-KOH, [pH 30 7.5], 5 mM EDTA, 0.1 mM sodium orthovanadate, 50 mM NaF, 0.1% Triton-X, 100 µg/ml PMSF, 2 µg/ml pepstatin, and 10 µg/ml each aprotonin and leupeptin), scraped, and spun at 15,000 x g for 10 min. Cleared lysate was precleared once with normal rabbit serum and twice with protein A- 35 sepharose (Pharmacia) before addition of antibodies (3 µl

- 32 -

of preimmune or immune rabbit serum). Immune complexes were harvested with protein A-sepharose, and beads were washed four times with lysis buffer before resuspension in denaturing sample buffer. Following SDS-PAGE, the gel 5 was enhanced for fluorography with Enlightening solution (DuPont), and dried down on absorbent paper.

Transfections and Immunofluorescence

BHK cells were plated the night before transfection at a density of  $10^4$  cells per 18mm round 10 coverslip (VWR Scientific). CaPO<sub>4</sub> precipitates were made by standard techniques and 18-20 h after transfection, cells were analyzed by immunofluorescence, as described by Heald et al. (Cell 74:463, 1993). Cells were fixed for 10 minutes at 3% formaldehyde and washed extensively 15 in PBS containing 0.1% NP40. Cells were reacted with primary and secondary antibodies for 30 minutes each. Nuclei were stained with Hoechst 33258 (1  $\mu$ g/ml) and mounted on slides with 90% glycerol, 0.2 M Tris (pH 8.0). The 9E10 anti-myc antibody (Evan et al., 1985) was 20 obtained as an ascites from the Harvard Cell Culture facility and used at 20  $\mu$ g/ml. RIP and  $\beta$ -galactosidase antisera were used at dilutions of 1:200. FITC anti-mouse and Texas Red anti-rabbit secondary antibodies were obtained from Jackson Immunoresearch. Cells were 25 examined with a Zeiss Axiophot™ fluorescent microscope.

$\beta$ -Galactosidase activity in cells was visualized by fixing cells with 0.5% glutaraldehyde for 15 min. followed by extensive washing in PBS containing 5 mM MgCl<sub>2</sub>. Cells were stained in PBS containing 20 mM 30 each K<sub>3</sub>Fe(CN)<sub>6</sub> and K<sub>4</sub>Fe(CN)<sub>6</sub>.3H<sub>2</sub>O, 1 mM MgCl<sub>2</sub>, and 1 mg/ml X-gal (5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside) until a suitable color developed, usually for 2-3 hours. To enumerate the fraction of blue cells which had undergone apoptotic changes, cells were transfected with one of the 35 four plasmid combinations described and fixed 16 h after

- 33 -

transfection. Blue cells were included for analysis only if their morphological status could be scored unambiguously.

Other embodiments of the invention are within  
5 the following claims.

- 34 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: President and Fellows of Harvard College
- (ii) TITLE OF INVENTION: CELL DEATH PROTEIN
- (iii) NUMBER OF SEQUENCES: 35
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Fish & Richardson P.C.
  - (B) STREET: 225 Franklin Street, Suite 3100
  - (C) CITY: Boston
  - (D) STATE: MA
  - (E) COUNTRY: USA
  - (F) ZIP: 02110-2804
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: PCT/US96/----
  - (B) FILING DATE: 18-APR-96
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/444,005
  - (B) FILING DATE: 18-MAY-95
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Clark, Paul T.
  - (B) REGISTRATION NUMBER: 30,164
  - (C) REFERENCE/DOCKET NUMBER: 00383/026001
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 617/542-5070
  - (B) TELEFAX: 617/542-8906
  - (C) TELEX: 200154

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 33 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGCGGGACGC GTAAGGAAGT ACAGAAAACA TGC

- 35 -

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGCGGGGCGG CCGCTCTAGA CCAAGCTTG GATTTC

36

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 33 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGCGGGGCGC GCTACCAAACG GTGGAAGTCC AAG

33

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGCGGGGCGG CCGCTGCCCG CAGGGCGCA GCCTCA

36

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 33 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- 36 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCGGGACGC GTAAGAAGCC CTTGTGCCCTG CAG

33

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCGGGGCGG CCGCTTTAAC TGGGCTTCAT CCCAGC

36

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGAAAGAATG GTAACAATGA AGCC

24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGCTTCATTG TTACCATTCT TTCG

24

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- 37 -

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATTCGTGCCA GCCAGAGCGG CATGGAGCAG AAGCTCATCT CAGAAGAAGA CCTCGCGTAA	60
GC	62

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 63 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGCCGCTTAC GCGAGGTCTT CTTCTGAGAT GAGCTTCTGC TCCATGCCGC TCTGGCTGGC	60
ACG	63

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCCAAGCTTG TTGGAGATTG TGACCAATC	29
---------------------------------	----

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 33 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCCGATCTGC AGGTCATGTA AGTAGCACAT GCC	33
--------------------------------------	----

- 38 -

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 60 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCCCCTCGAGT TAGAGGTCTT CTTCTGAGAT GAGCTTTGC TCTTTCTTA AACTTGCCAC 60

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 2268 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CATACTGAGC AAGAACAAA AGTGGTGTGT TGGAGATTCT GAGCAATCAA AATGCAACCA	60
GACATGTCCT TGGACAATAT TAAGATGGCA TCCAGTGACC TGCTGGAGAA GACAGACCTA	120
GACACGGAG GCTTCGGGAA GGTGTCCTTG TGTTACCACA GAAGCCATGG ATTTGTCATC	180
CTGAAAAAAG TATACACAGG GCCCAACCGC GCTGAGTACA ATGAGGTTCT CTTGGAAGAG	240
GGGAAGATGA TGCACAGACT GAGACACAGT CGAGTGGTGA AGCTACTGGG CATCATCATA	300
GAAGAAGGGA ACTATTCGCT GGTGATGGAG TACATGGAGA AGGGCAACCT GATGCCACGTG	360
CTAAAGACCC AGATAGATGT CCCACTTTCA TTGAAAGGAA GGATAATCGT GGAGGCCATA	420
GAAGGCATGT GCTACTTACA TGACAAAGGT GTGATACACA AGGACCTGAA GCCTGAGAAT	480
ATCCTCGTTG ATCGTGAATT TCACATTAAG ATAGCCGATC TTGGTGTGGC TTCCTTTAAG	540
ACATGGAGCA AACTGACTAA GGAGAAAGAC AACAAAGCAGA AAGAAGTGAG CAGCACCACT	600
AAGAAGAACCA ATGGTGGTAC CCTTTACTAC ATGGCACCCG AACACCTGAA TGACATCAAT	660
GCAAAGCCCA CGGAGAAAGTC GGACGTGTAC AGCTTTGGCA TTGTCCTTTG GGCAATATTT	720
GCAAAAAAAGG AGCCCTATGA GAATGTCATC TGTACTGAGC AGTTCGTGAT CTGCATAAAA	780
TCTGGGAACCA GGCAAATGT AGAGGAAATC CTTGAGTACT GTCCAAGGGA GATCATCAGC	840
CTCATGGAGC GGTGCTGGCA GGCGATCCCA GAAGACAGGC CAACATTCT TGGCATTGAA	900
GAAGAATTAA GGCTTTTTA CTTAAGTCAT TTTGAAGAAT ATGTAGAAGA GGATGTGGCA	960

- 39 -

AGTTTAAAGA AAGAGTATCC AGATCAAAGC CCAGTGCTGC AGAGAATGTT TTCACTGCAG	1020
CATGACTGTG TACCCCTTACC TCCGAGCAGG TCAAATTCAAG AACAAACCTGG ATCGCTGCAC	1080
AGTTCCCAGG GGCTCCAGAT GGGTCCTGTG GAGGAGTCCT GGTTTCTTC CTCCCCAGAG	1140
TACCCACAGG ACGAGAACATGA TCGCAGTGTG CAGGCTAACGC TGCAAGAGGA AGCCAGCTAT	1200
CATGCTTTG GAATATTTGC AGAGAAACAG ACAAAACCGC AGCCAAGGCA GAATGAGGCT	1260
TACAACAGAG AGGAGGAAAG GAAACGAAGG GTCTCTCATG ACCCCTTTC ACAGCAGAGA	1320
GCTCGTGAGA ATATTAAGAG TGCAGGAGCA AGAGGTCATT CTGATCCCAG CACAACGAGT	1380
CGTGGAAATTG CAGTGCRAACA GCTGTCATGG CCAGGCCACCC AAACAGTTG GAACAATGGA	1440
TTGTATAATC AGCATGGATT TGGAACTACA GGTACAGGAG TTTGGTATCC GCCAAATCTA	1500
AGCCAAATGT ATAGTACTTA TAAAACCTCA GTGCCAGAGA CCAACATACC GGGAAAGCACA	1560
CCCACCATGC CATACTTCTC TGGGCCAGTA GCAGATGACC TCATAAAATA TACTATATTC	1620
AATAGTTCTG GTATTCAAGAT TGGAAACCCAC AATTATATGG ATGTTGGACT GAATTCAACAA	1680
CCACCAAACA ATACTTGCAA AGAAGAGTCG ACTTCCAGAC ACCAAGCCAT CTTTGATAAC	1740
ACCACTAGTC TGACTGATGA ACACCTGAAC CCTATCAGGG AAAACCTGGG AAGGCAGTGG	1800
AAAAACTGTG CCCGCAAGCT GGGCTTCACT GAGTCTCAGA TCGATGAAAT CGACCATGAC	1860
TATGAAAGAG ATGGACTGAA AGAGAAAGTT TACCAAATGC TTCAGAACTG GCTGATGCGG	1920
GAAGGCCACCA AAGGGGCCAC AGTGGGAAAG TTGGCCAGG CACTTCACCA ATGTTGCAGG	1980
ATAGACCTGC TGAACCACTT GATTGTCGCC AGCCAGAGCT AAGCCTGGC AGGCTCTGGC	2040
AGTGGGAAGC AAACTATTTG TCTGGTGCAC AAACCCCGTT TGCCCACTAG CCTTCAGAAC	2100
TCTATCTCAG CATGAGCTCT GCATTGAGC ACACAGGGTC ATGCAGTTG GAACTGGTGG	2160
ATGGGAAGAG AAATCTGAAG CCCACAGTGA TTCTTCAGAA CATCCAAGCA TAAAGACCGC	2220
TGAATGAATG GTCGGTCCAT GACCAGTAGG AGAAAAAAA AAAAAAAG	2268

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 656 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met	Gln	Pro	Asp	Met	Ser	Leu	Asp	Asn	Ile	Lys	Met	Ala	Ser	Ser	Asp
1						5				10				15	

- 40 -

Leu Leu Glu Lys Thr Asp Leu Asp Ser Gly Gly Phe Gly Lys Val Ser  
 20 25 30

Leu Cys Tyr His Arg Ser His Gly Phe Val Ile Leu Lys Lys Val Tyr  
 35 40 45

Thr Gly Pro Asn Arg Ala Glu Tyr Asn Glu Val Leu Leu Glu Gly  
 50 55 60

Lys Met Met His Arg Leu Arg His Ser Arg Val Val Lys Leu Leu Gly  
 65 70 75 80

Ile Ile Ile Glu Glu Gly Asn Tyr Ser Leu Val Met Glu Tyr Met Glu  
 85 90 95

Lys Gly Asn Leu Met His Val Leu Lys Thr Gln Ile Asp Val Pro Leu  
 100 105 110

Ser Leu Lys Gly Arg Ile Ile Val Glu Ala Ile Glu Gly Met Cys Tyr  
 115 120 125

Leu His Asp Lys Gly Val Ile His Lys Asp Leu Lys Pro Glu Asn Ile  
 130 135 140

Leu Val Asp Arg Asp Phe His Ile Lys Ile Ala Asp Leu Gly Val Ala  
 145 150 155 160

Ser Phe Lys Thr Trp Ser Lys Leu Thr Lys Glu Lys Asp Asn Lys Gln  
 165 170 175

Lys Glu Val Ser Ser Thr Thr Lys Lys Asn Asn Gly Gly Thr Leu Tyr  
 180 185 190

Tyr Met Ala Pro Glu His Leu Asn Asp Ile Asn Ala Lys Pro Thr Glu  
 195 200 205

Lys Ser Asp Val Tyr Ser Phe Gly Ile Val Leu Trp Ala Ile Phe Ala  
 210 215 220

Lys Lys Glu Pro Tyr Glu Asn Val Ile Cys Thr Glu Gln Phe Val Ile  
 225 230 235 240

Cys Ile Lys Ser Gly Asn Arg Pro Asn Val Glu Glu Ile Leu Glu Tyr  
 245 250 255

Cys Pro Arg Glu Ile Ile Ser Leu Met Glu Arg Cys Trp Gln Ala Ile  
 260 265 270

Pro Glu Asp Arg Pro Thr Phe Leu Gly Ile Glu Glu Phe Arg Pro  
 275 280 285

Phe Tyr Leu Ser His Phe Glu Glu Tyr Val Glu Glu Asp Val Ala Ser  
 290 295 300

Leu Lys Lys Glu Tyr Pro Asp Gln Ser Pro Val Leu Gln Arg Met Phe  
 305 310 315 320

Ser Leu Gln His Asp Cys Val Pro Leu Pro Pro Ser Arg Ser Asn Ser  
 325 330 335

Glu Gln Pro Gly Ser Leu His Ser Ser Gln Gly Leu Gln M t Gly Pro  
 340 345 350

- 41 -

Val Glu Glu Ser Trp Phe Ser Ser Ser Pro Glu Tyr Pro Gln Asp Glu  
355 360 365

Asn Asp Arg Ser Val Gln Ala Lys Leu Gln Glu Glu Ala Ser Tyr His  
370 375 380

Ala Phe Gly Ile Phe Ala Glu Lys Gln Thr Lys Pro Gln Pro Arg Gln  
385 390 395 400

Asn Glu Ala Tyr Asn Arg Glu Glu Arg Lys Arg Arg Val Ser His  
405 410 415

Asp Pro Phe Ala Gln Gln Arg Ala Arg Glu Asn Ile Lys Ser Ala Gly  
420 425 430

Ala Arg Gly His Ser Asp Pro Ser Thr Thr Ser Arg Gly Ile Ala Val  
435 440 445

Gln Gln Leu Ser Trp Pro Ala Thr Gln Thr Val Trp Asn Asn Gly Leu  
450 455 460

Tyr Asn Gln His Gly Phe Gly Thr Thr Gly Thr Gly Val Trp Tyr Pro  
465 470 475 480

Pro Asn Leu Ser Gln Met Tyr Ser Thr Tyr Lys Thr Pro Val Pro Glu  
485 490 495

Thr Asn Ile Pro Gly Ser Thr Pro Thr Met Pro Tyr Phe Ser Gly Pro  
500 505 510

Val Ala Asp Asp Leu Ile Lys Tyr Thr Ile Phe Asn Ser Ser Gly Ile  
515 520 525

Gln Ile Gly Asn His Asn Tyr Met Asp Val Gly Leu Asn Ser Gln Pro  
530 535 540

Pro Asn Asn Thr Cys Lys Glu Glu Ser Thr Ser Arg His Gln Ala Ile  
545 550 555 560

Phe Asp Asn Thr Thr Ser Leu Thr Asp Glu His Leu Asn Pro Ile Arg  
565 570 575

Glu Asn Leu Gly Arg Gln Trp Lys Asn Cys Ala Arg Lys Leu Gly Phe  
580 585 590

Thr Glu Ser Gln Ile Asp Glu Ile Asp His Asp Tyr Glu Arg Asp Gly  
595 600 605

Leu Lys Glu Lys Val Tyr Gln Met Leu Gln Lys Trp Leu Met Arg Glu  
610 615 620

Gly Thr Lys Gly Ala Thr Val Gly Lys Leu Ala Gln Ala Leu His Gln  
625 630 635 640

Cys Cys Arg Ile Asp Leu Leu Asn His Leu Ile Arg Ala Ser Gln Ser  
645 650 655

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 2137 base pairs  
(B) TYPE: nucleic acid

- 42 -

(C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ATGCAACCAAG ACATGTCCTT GAATGTCATT AAGATGAAAT CCAGTGACTT CCTGGAGAGT	60
GCAGAACTGG ACAGCGGAGG CTTCGGGAAAG GTGTCTCTGT GTTTCCACAG AACCCAGGGA	120
CTCATGATCA TGAAAACAGT GTACAAGGGG CCCAAGTGCA TTGAGCACAA CGAGGCCCTC	180
TTGGAGGAGG CGAAGATGAT GAACAGACTG AGACACAGCC GGGTGGTGAA GCTCCTGGGC	240
GTCATCATAG AGGAAGGGAA GTACTCCCTG GTGATGGAGT ACATGGAGAA GGGCAACCTG	300
ATGCACGTGC TGAAAGCCGA GATGAGTACT CCGCTTTCTG TAAAAGGAAG GATAATTITG	360
GAAATCATTG AAGGAATGTG CTACTTACAT GGAAAAGGCG TGATACACAA GGACCTGAAG	420
CCTGAAAATA TCCTTGTGA TAATGACTTC CACATTAAGA TCGCAGACCT CGGCCTTGCC	480
TCCTTTAAGA TGTGGAGCAA ACTGAATAAT GAAGAGCACA ATGAGCTGAG GGAAGTGGAC	540
GGCACCGCTA AGAAGAATGG CGGCACCCCTC TACTACATGG CGCCCCAGCA CCTGAATGAC	600
GTCAACGCAA AGCCCACAGA GAAGTCGGAT GTGTACAGCT TTGCTGTAGT ACTCTGGCG	660
ATATTTGCAA ATAAGGAGCC ATATGAAAAT GCTATCTGTG AGCAGCAGTT GATAATGTGC	720
ATAAAATCTG GGAACAGGCC AGATGTGGAT GACATCACTG AGTACTGCCA AAGAGAAATT	780
ATCAGTCTCA TGAAGCTCTG CTGGGAAGCG AATCCGGAAG CTCGGCCGAC ATTTCTGGC	840
ATTGAAGAAA AATTTAGGCC TTTTTATTTA AGTCATTTAG AAGAAAGTGT AGAAGAGGAC	900
GTGAAGAGTT TAAAGAAAGA GTATTCAAAC GAAAATGCAG TTGTGAAGAG AATGCAGTCT	960
CITCAACTTG ATTGTGTGGC AGTACCTTCA AGCCGGTCRA ATTCAAGCCAC AGAACAGGCT	1020
GGTTCACTGC ACAGTTCCA GGGACTTGGG ATGGGTCTGT TGGAGGAGTC CTGGTTGGT	1080
CCTTCCCTGG AGCACCCACA AGAAGAGAAAT GAGCCCAGCC TGCAGAGTAA ACTCCAAGAC	1140
GAAGCCAAC ACCATCTTTA TGGCAGCCGC ATGGACAGGC AGACGAAACA GCAGCCCAGA	1200
CAGAATGTGG CTTACAACAG AGAGGAGGAA AGGAGACGCA GGGTCTCCCA TGACCCCTTT	1260
GCACAGCAA GACCTTACGA GAATTTTCAG AATACAGAGG GAAAAGGCAC TGTATTCC	1320
AGTGCAGCCA GTCATGGTAA TGCAGTGCAC CAGCCATCAG GGCTCACCAG CCAACCTCAA	1380
GTACTGTATC AGAACAAATGG ATTATATAGC TCACATGGCT TTGGAACAAG ACCACTGGAT	1440
CCAGGAACAG CAGGTCCAG AGTTGGTAC AGGCCAATTC CAAGTCATAT GCCTAGTCTG	1500
CATAATATCC CAGTGCCTGA GACCAACTAT CTAGGAAATT CTCCCACCAT GCCATTAGC	1560
TCCCTGCCAC CAACAGATGA ATCTATAAAA TATACCATAT ACAATAGTAC TGGCATTAGC	1620

- 43 -

ATTGGAGCCT ACAATTATAT GGAGATTGGT GGGACCGAGTT CATCACTACT AGACAGCACA	1680
AATACTGAAC TCAAAGAAGA GCCAGCTGCT AAGTACCAAG CTATCTTGTA TAATACCACT	1740
AGTCTGACGG ATAAACACCT GGACCCAATC AGGGAAAATC TGGGAAAGCA CTGGAAAAAC	1800
TGTGCCGTA AACTGGGCTT CACACAGTCT CAGATTGATG AAATTGACCA TGACTATGAG	1860
CGAGATGGAC TGAAAGAAAA GGTTTACCAAG ATGCTCCAAA AGTGGGTGAT GAGGGAAGGC	1920
ATAAAGGGAG CCACGGTGGG GAAGCTGGCC CAGGCGCTCC ACCAGTGTTC CAGGATCGAC	1980
CTTCTGAGCA CCTTGATTTA CGTCAGCCAG AACTAACCCCT GGATGGGCTA CGGCAGCTGA	2040
AGTGGACGCC TCACITTAGTG GATAACCCC AAAAGTTGGC TGCCTCAGAG CATTAGAAT	2100
TCTGTCCCTCA CTGATAGGGG TTCTGTGTCT GCAGAAA	2137

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 709 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Gln Pro Asp Met Ser Leu Asn Val Ile Lys Met Lys Ser Ser Asp	
1 5 10 15	
Phe Leu Glu Ser Ala Glu Leu Asp Ser Gly Gly Phe Gly Lys Val Ser	
20 25 30	
Leu Cys Phe His Arg Thr Gln Gly Leu Met Ile Met Lys Thr Val Tyr	
35 40 45	
Lys Gly Pro Asn Cys Ile Glu His Asn Glu Ala Leu Leu Glu Glu Ala	
50 55 60	
Lys Met Met Asn Arg Leu Arg His Ser Arg Val Val Lys Leu Leu Gly	
65 70 75 80	
Val Ile Ile Glu Glu Gly Lys Tyr Ser Leu Val Met Glu Tyr Met Glu	
85 90 95	
Lys Gly Asn Leu Met His Val Leu Lys Ala Glu Met Ser Thr Pro Leu	
100 105 110	
Ser Val Lys Gly Arg Ile Ile Leu Glu Ile Ile Glu Gly Met Cys Tyr	
115 120 125	
Leu His Gly Lys Gly Val Ile His Lys Asp Leu Lys Pro Glu Asn Ile	
130 135 140	
Leu Val Asp Asn Asp Phe His Ile Lys Ile Ala Asp Leu Gly Leu Ala	
145 150 155 160	

- 44 -

Ser Phe Lys Met Trp Ser Lys Leu Asn Asn Glu Glu His Asn Glu Leu  
165 170 175

Arg Glu Val Asp Gly Thr Ala Lys Lys Asn Gly Gly Thr Leu Tyr Tyr  
180 185 190

Met Ala Pro Glu His Leu Asn Asp Val Asn Ala Lys Pro Thr Glu Lys  
195 200 205

Ser Asp Val Tyr Ser Phe Ala Val Val Leu Trp Ala Ile Phe Ala Asn  
210 215 220

Lys Glu Pro Tyr Glu Asn Ala Ile Cys Glu Gln Gln Leu Ile Met Cys  
225 230 235 240

Ile Lys Ser Gly Asn Arg Pro Asp Val Asp Asp Ile Thr Glu Tyr Cys  
245 250 255

Pro Arg Glu Ile Ile Ser Leu Met Lys Leu Cys Trp Glu Ala Asn Pro  
260 265 270

Glu Ala Arg Pro Thr Phe Pro Gly Ile Glu Glu Lys Phe Arg Pro Phe  
275 280 285

Tyr Leu Ser Gln Leu Glu Glu Ser Val Glu Glu Asp Val Lys Ser Leu  
290 295 300

Lys Lys Glu Tyr Ser Asn Glu Asn Ala Val Val Lys Arg Met Gln Ser  
305 310 315 320

Leu Gln Leu Asp Cys Val Ala Val Pro Ser Ser Arg Ser Asn Ser Ala  
325 330 335

Thr Glu Gln Ala Gly Ser Leu His Ser Ser Gln Gly Leu Gly Met Gly  
340 345 350

Pro Val Glu Glu Ser Trp Phe Gly Pro Ser Leu Glu His Pro Gln Glu  
355 360 365

Glu Asn Glu Pro Ser Leu Gln Ser Lys Leu Gln Asp Glu Ala Asn Tyr  
370 375 380

His Leu Tyr Gly Ser Arg Met Asp Arg Gln Thr Lys Gln Gln Pro Arg  
385 390 395 400

Gln Asn Val Ala Tyr Asn Arg Glu Glu Glu Arg Arg Arg Arg Val Ser  
405 410 415

His Asp Pro Phe Ala Gln Gln Arg Pro Tyr Glu Asn Phe Gln Asn Thr  
420 425 430

Glu Gly Lys Gly Thr Val Tyr Ser Ser Ala Ala Ser His Gly Asn Ala  
435 440 445

Val His Gln Pro Ser Gly Leu Thr Ser Gln Pro Gln Val Leu Tyr Gln  
450 455 460

Asn Asn Gly Leu Tyr Ser Ser His Gly Phe Gly Thr Arg Pro Leu Asp  
465 470 475 480

Pro Gly Thr Ala Gly Pro Arg Val Trp Tyr Arg Pro Ile Pro Ser His  
485 490 495

- 45 -

Met Pro Ser Leu His Asn Ile Pro Val Pro Glu Thr Asn Tyr Leu Gly		
500	505	510
Asn Ser Pro Thr Met Pro Phe Ser Ser Leu Pro Pro Thr Asp Glu Ser		
515	520	525
Ile Lys Tyr Thr Ile Tyr Asn Ser Thr Gly Ile Gln Ile Gly Ala Tyr		
530	535	540
Asn Tyr Met Glu Ile Gly Gly Thr Ser Ser Ser Leu Leu Asp Ser Thr		
545	550	555
Asn Thr Asn Phe Lys Glu Glu Pro Ala Ala Lys Tyr Gln Ala Ile Phe		
565	570	575
Asp Asn Thr Thr Ser Leu Thr Asp Lys His Leu Asp Pro Ile Arg Glu		
580	585	590
Asn Leu Gly Lys His Trp Lys Asn Cys Ala Arg Lys Leu Gly Phe Thr		
595	600	605
Gln Ser Gln Ile Asp Glu Ile Asp His Asp Tyr Glu Arg Asp Gly Leu		
610	615	620
Lys Glu Lys Val Tyr Gln Met Leu Gln Lys Trp Val Met Arg Glu Gly		
625	630	635
Ile Lys Gly Ala Thr Val Gly Lys Leu Ala Gln Ala Leu His Gln Cys		
645	650	655
Ser Arg Ile Asp Leu Leu Ser Ser Leu Ile Tyr Val Ser Gln Asn Pro		
660	665	670
Trp Met Gly Tyr Gly Ser Ser Gly Arg Leu Thr Trp Ile Thr Pro Glu		
675	680	685
Ser Trp Leu Pro Gln Ser Ile Gln Asn Ser Val Leu Thr Asp Arg Gly		
690	695	700
Ser Val Ser Ala Glu		
705		

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 38 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser Arg His Gln Ala Ile Phe Asp Asn Thr Thr Ser Leu Thr Asp Glu		
1	5	10
		15

- 46 -

His Leu Asn Pro Ile Arg Glu Asn Leu Gly Arg Gln Trp Lys Asn Cys  
20 25 30

Ala Arg Lys Leu Gly Phe  
35

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 38 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Ala Lys Tyr Gln Ala Ile Phe Asp Asn Thr Thr Ser Leu Thr Asp Lys  
1 5 10 15

His Leu Asp Pro Ile Arg Glu Asn Leu Gly Lys His Trp Lys Asn Cys  
20 25 30

Ala Arg Lys Leu Gly Phe  
35

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ser Lys Tyr Ile Pro Arg Ile Ala Glu Asp Met Thr Ile Gln Glu Ala  
1 5 10 15

Lys Lys Phe Ala Arg Glu Asn Asn Ile  
20 25

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- 47 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ser Lys Tyr Ile Thr Thr Ile Ala Gly Val Met Thr Leu Ser Gln Val  
1 5 10 15  
Lys Gly Phe Val Arg Lys Asn Gly Val  
20 25

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Lys Trp Glu Asp Ser Ala His Pro Gln Arg Pro Asp Asn Ala Asp Leu  
1 5 10 15  
Ala Ile Leu Tyr Ala Val Val Asp Gly Val Pro Pro Ala Arg Trp Lys  
20 25 30  
Glu Phe Met Arg Phe Met Gly Leu  
35 40

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp Thr Asp Asp  
1 5 10 15  
Pro Ala Thr Leu Tyr Ala Val Val Glu Asn Val Pro Pro Leu Arg Trp  
20 25 30  
Lys Glu Phe Val Arg Arg Leu Gly Leu  
35 40

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

- 48 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Thr Glu Ser Gln Ile Asp Glu Ile Asp His Asp Tyr Glu Arg Asp Gly  
1 5 10 15  
Leu Lys Glu Lys Val Tyr Gln Met Leu Gln Lys Trp Leu Met Arg Glu  
20 25 30  
Gly Thr Lys Gly Ala Thr Val Gly Lys Leu  
35 40

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 42 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Thr Gln Ser Gln Ile Asp Glu Ile Asp His Asp Tyr Glu Arg Asp Gly  
1 5 10 15  
Leu Lys Glu Lys Val Tyr Gln Met Leu Gln Lys Trp Val Met Arg Glu  
20 25 30  
Gly Ile Lys Gly Ala Thr Val Gly Lys Leu  
35 40

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 40 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Lys Glu Gly Lys Ile Asp Glu Ile Met His Asp Ser Ile Gln Asp Thr  
1 5 10 15  
Ala Glu Gln Lys Val Gln Leu Leu Cys Trp Tyr Gln Ser His Gly  
20 25 30  
Lys Ser Asp Ala Tyr Gln Asp Leu  
35 40

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:

- 49 -

- (A) LENGTH: 40 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Asn	Glu	Ala	Lys	Ile	Asp	Glu	Ile	Lys	Asn	Asp	Asn	Val	Gln	Asp	Thr
1				5					10					15	
Ala	Glu	Gln	Lys	Val	Gln	Leu	Leu	Arg	Asn	Trp	His	Gln	Leu	His	Gly
				20				25					30		
Lys	Lys	Glu	Ala	Tyr	Asp	Thr	Leu								
				35			40								

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Ser	Glu	His	Glu	Ile	Glu	Arg	Leu	Glu	Met	Gln	Asn	Gly	Arg	Cys	Leu
1					5				10					15	
Arg	Glu	Ala	Gln	Tyr	Ser	Met	Leu	Glu	Ala	Trp	Arg	Arg	Arg	Thr	Pro
				20				25					30		
Arg	His	Glu	Asp	Thr	Leu	Glu	Val	Val							
				35			40								

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Ser	Asp	His	Glu	Ile	Asp	Arg	Leu	Glu	Leu	Gln	Asn	Gly	Arg	Cys	Leu
1					5				10					15	

- 50 -

Arg Glu Ala Gln Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Arg Thr Pro  
20 25 30

Arg Arg Glu Ala Thr Leu Glu Leu Leu  
35 40

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 23 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Ala Gln Ala Leu His Gln Cys Cys Arg Ile Asp Leu Leu Asn His Leu  
1 5 10 15

Ile Leu Arg Ala Ser Gln Ser  
20

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Ala Gln Ala Leu His Gln Cys Ser Arg Ile Asp Leu Leu Ser Ser Leu  
1 5 10 15

Ile Tyr Val Ser Gln Asn  
20

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 41 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

- 51 -

Ile Lys Gly Leu Lys Lys Ala Glu Cys Arg Arg Thr Leu Asp Lys Phe  
1 5 10 15  
Gln Asp Met Val Gln Lys Asp Leu Gly Lys Ser Thr Pro Asp Thr Gly  
20 25 30  
Asn Glu Asn Glu Gly Gln Cys Leu Glu  
35 40

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 41 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Ile Lys Asp Leu Lys Lys Ala Asn Leu Cys Thr Leu Ala Glu Lys Ile  
1 5 10 15  
Gln Thr Ile Ile Leu Lys Asp Ile Thr Ser Asp Ser Glu Asn Ser Asn  
20 25 30  
Phe Arg Asn Glu Ile Gln Ser Leu Val  
35 40

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 33 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant  
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Gly Leu Val Leu Ser Lys Met Asn Leu Ala Gly Cys Leu Glu Asn Ile  
1 5 10 15  
Leu Glu Ala Leu Arg Asn Pro Ala Pro Ser Ser Thr Thr Arg Leu Pro  
20 25 30  
Arg

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 35 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: not relevant

- 52 -

(D) TOPOLOGY: lin ar

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly Cys Leu Glu Asp Ile  
1 5 10 15

Glu Glu Ala Leu Cys Cys Gly Pro Ala Ala Leu Pro Pro Ala Pro Ser  
20 25 30

Leu Leu Arg  
35

Claims

We claim:

1. An isolated receptor interacting protein (RIP), comprising a death domain in its carboxy terminal region and a kinase domain in its amino terminal region, said RIP being capable of interacting with a Fas/APO-1 intracellular domain, and being capable of promoting apoptosis in a eukaryotic cell.
2. The RIP of claim 1, said RIP being a human protein.
3. The RIP of claim 2, said RIP comprising an amino acid sequence consisting of (SEQ ID NO:17).
4. The RIP of claim 1, said RIP being a murine protein.
5. The RIP of claim 4, said RIP comprising an amino acid sequence consisting of (SEQ ID NO:15).
6. An isolated polypeptide comprising the death domain of an RIP.
7. An isolated polypeptide comprising the kinase domain of an RIP.
8. An isolated DNA molecule comprising a nucleotide sequence encoding an RIP, said RIP comprising a death domain in its carboxy terminal region and a kinase domain in its amino terminal region, said RIP being capable of interacting with a Fas intracellular domain, and said RIP being capable of promoting apoptosis in a eukaryotic cell.

- 54 -

9. The isolated DNA molecule of claim 8,  
wherein said nucleotide sequence encodes a human RIP.

10. The isolated DNA molecule of claim 8,  
wherein said nucleotide sequence encodes a murine RIP.

5 11. An isolated DNA molecule comprising a  
nucleotide sequence encoding an RIP death domain.

12. An isolated DNA molecule comprising a  
nucleotide sequence encoding an RIP kinase domain.

10 13. A transformed host cell comprising a  
recombinant DNA molecule encoding an RIP.

14. The transformed host cell of claim 13,  
said transformed host cell being a cultured cell of a  
metazoan animal.

15 15. The transformed host cell of claim 13,  
said transformed host cell being prokaryotic.

16. A polyclonal antibody preparation obtained  
by immunizing an animal with an isolated protein  
comprising an RIP death domain, said polyclonal antibody  
preparation having binding activity directed to the RIP  
intracellular domain.

20 17. The polyclonal antibody preparation of  
claim 16, wherein said binding activity directed to the  
RIP death domain is substantially greater than the  
binding activity directed to any member of the NGFR/TNFR  
protein family.

- 55 -

18. A monoclonal antibody that reacts with an RIP death domain.

19. The monoclonal antibody of claim 18, whose binding directed to the RIP intracellular domain is 5 substantially greater than the binding activity directed to any member of the NGFR/TNFR protein family.

20. A cellular screening method for identifying chemical compounds capable of inhibiting RIP-dependent promotion of apoptosis, said method comprising 10 the steps of:

(a) expressing recombinant RIP in a transformed host cell;

(b) contacting said transformed host cell with an inhibitor candidate compound;

15 (c) detecting morphological or biochemical indicia of apoptosis in said transformed host cell at an appropriate time interval following expression of recombinant RIP; and

(d) comparing the detection in step (c) with 20 the corresponding detections from one or more appropriate experimental controls.

21. The method of claim 20, wherein said recombinant RIP is expressed under the control of an inducible promoter.

25 22. The method of claim 20, wherein step (b) precedes step (a).

23. A cell-free screening method for identifying chemical compounds capable of inhibiting RIP-dependent promotion of apoptosis, said method comprising 30 the steps of:

- 56 -

- (a) contacting, in the presence of an inhibitor candidate compound, a protein comprising the death domain of RIP with an RIP interaction partner;
- (b) detecting a specific interaction, in the presence of an inhibitor candidate compound, between said protein comprising the death domain of RIP and said RIP interaction partner;
- (c) contacting, in the absence of said inhibitor candidate compound, said protein comprising the death domain of RIP with said RIP interaction partner;
- (d) detecting a specific interaction, in the absence of said inhibitor candidate compound, between said protein comprising the death domain of RIP and said RIP interaction partner; and
- (e) comparing the detection in step (b) and the detection in step (d).

24. The method of claim 23, wherein said RIP interaction partner is Fas/APO-1.

25. The method of claim 23, wherein said RIP interaction partner is inserted into a biological membrane.

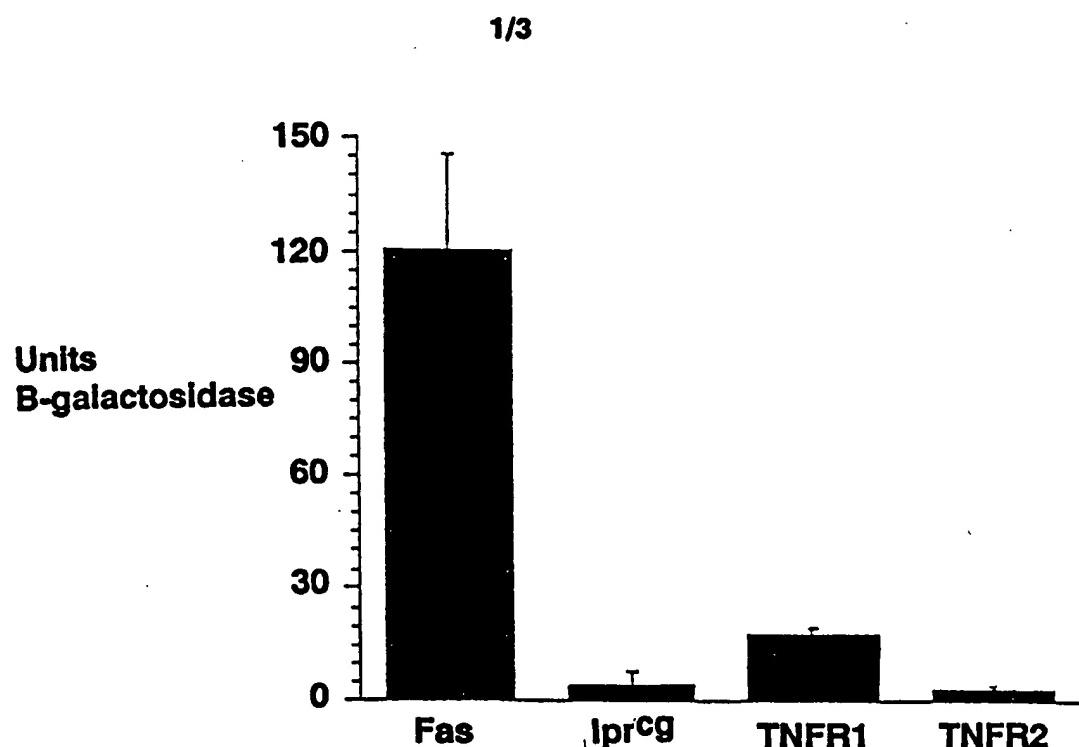
26. A cellular screening method for identifying chemical compounds capable of inducing RIP expression and apoptosis resulting therefrom, said method comprising the steps of:

- (a) culturing a eukaryotic cell;
- (b) contacting said eukaryotic cell in culture with a RIP-inducing compound candidate;
- (c) detecting morphological or biochemical indicia of apoptosis in said transformed host cell at an appropriate time interval following expression of recombinant RIP; and

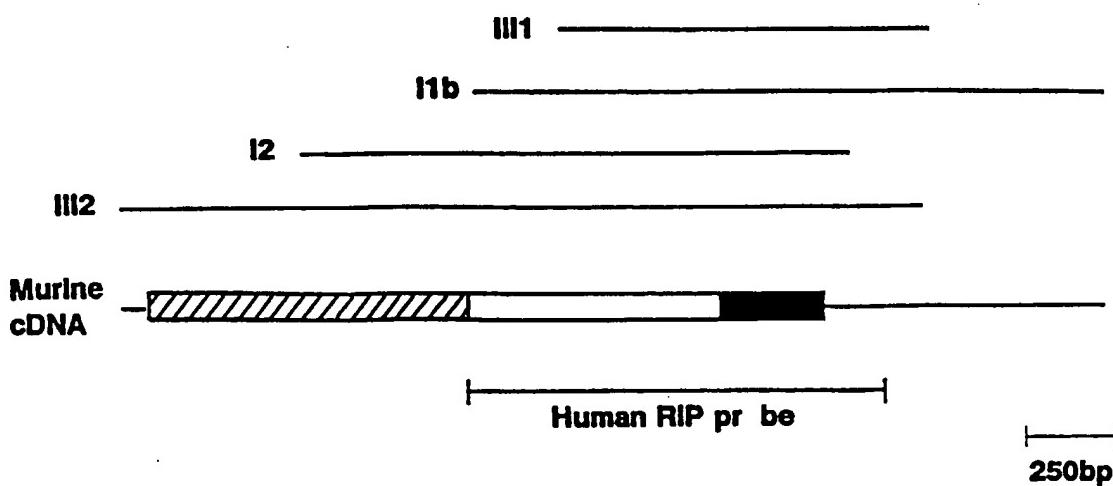
- 57 -

(d) comparing the detection in step (c) with the corresponding detections from one or more appropriate experimental controls.

27. A method of producing, for introduction  
5 into a patient undergoing gene therapy, cells that can be induced to undergo apoptosis upon administration of an exogenous agent to said patient.



**FIG. 1**



**FIG. 2**

SUBSTITUTE SHEET (RULE 26)

2/3

(SEQ ID NO:15)

1 MQPDMSLDNIKMASSDLLEKTDLDSGGFGKVSLCYHRSHG  
 41 FVILKKVYTGPNRAEYNNEVLLEEGKMMHRLRHSRVVKLLG  
 81 IIIIEEGNYSLVMEYMEKGNLHMVLKTQIDVPLSLKGRIIV  
 121 EAIEGMCYLHDKGVIHKDLKPENILVDRDFHIKIADLGVA  
 161 SFKTWSKLTKEKDNKQKEVSSTTKNNNGTLYYMAPEHLN  
 201 DINAKPTEKSDVYSFGIVLWAI~~F~~AKKEPYENVICTEQFVI  
 241 CIKSGNRPNV~~E~~EILEYCPREIISLMERCWQAIPEDRPTFL  
 281 GIEEEFRPFYLSHFE~~E~~YVEEDVASLKKEYPDQSPV~~L~~QRMF  
 1 --K-----SNENA-VKR-Q  
 321 SLQHDCVPLPPSRNS..EQPGSLHSSQGLQMG~~P~~VEESWF  
 21 ---L---AV-S----AT-----G-----  
 359 SSSPEY~~P~~QDENDRSVQAKLQEEASYHAFGI~~F~~AEKQT~~K~~PQ~~P~~  
 61 AP-L-H--E--EP-L-S--D--N--LY-SRM~~D~~R---Q--  
 399 RQNEAYNREERKRRVSHDPFAQQ~~R~~AREN~~I~~K~~S~~AGARGHSD  
 101 ---V-----R-----PY--FQNTEGK-TVY  
 439 PTTTSRGIAVQQ..LSWPATQTVWNNGLYNQHGF~~G~~T  
 141 S-AA-H-N--H-PSG-TSQPQVLYQ----SS----RPL  
 473 . . . TGTGVWYPPNLSQMYSTYKTPV~~P~~ETNIPGSTPTM~~P~~Y  
 181 DPGTA-PR---R-IP-H-P-LHNI----YL-NS----F  
 509 FSGPVADDLIKYTIFNSSGIQIGNHNYMDVGLNS...Q~~P~~  
 221 S-L-PT-ES----Y--T----AY---EI-GT-SSL~~L~~DS  
 545 PNNTCKEESTSRHQAI~~F~~DNTTSLTDEHLNPIREN~~L~~GRQWK  
 261 T-TNF---PAAKY----K--D----KH--  
 585 NCARKLGFTESQIDEIDHDYERDGLKEK~~V~~YQMLQKWL~~M~~R~~E~~  
 301 ---Q-----.V-----.  
 625 GTKGATVGKLAQALHQCCRIDL~~N~~H~~L~~IRASQS  
 341 -I-----S----SS--YV--N

**FIG. 3**

SUBSTITUTE SHEET (RULE 26)

3/3

**SUBSTITUTE SHEET (RULE 28)**

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/05386

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :435/6, 7.1, 69.1, 71.2, 240.2; 530/350, 388.15; 514/44; 536/23.5

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 69.1, 71.2, 240.2; 530/350, 388.15; 514/44; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Sequence Searches - MasPar; APS and STN (Medline and CaPlus). Search Terms used: Programmed Cell Death; PCD and DNA; Apoptosis and DNA and protein and cell death; etc.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cell, Volume 74, issued 10 September 1993, Tartaglia et al., " A Novel Domain within the 55kd TNF Receptor Signals Cell Death". Pages 845-850. Especially see page 846, fig. 1, and page 847-48, Table 1 and fig. 3.	1-7
Y	Nature. Vol. 372, Issued 22/29 December 1994, Hubbard et al., " Crystal Structure of the Tyrosine Kinase Domain of the Human Insulin Receptor". See abstract and sequence alignment in fig.3.	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
24 JUNE 1996	15 JUL 1996

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>[Signature]</i> for TEKCHAND SAIDHA Telephone No. (703) 308-0196
---	---

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/05386

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY. Vol. 270, No. 1, issued 06 January 1995, Boldin et al., "Self-association of the 'Death Domains' of the p55 Tumor Necrosis Factor (TNF) Receptor and Fas/APO1 Prompts Signaling for TNF and Fas/APO1 Effects. Especially see Table 1.	1-15
X ---	US 5,360,893 A (G.P. Owens et al.) 01 November 1994, columns 27-28, claims 1 & 2; column 2, lines 5-44.	11 -----
Y		20-27
Y	Science, Vol. 245, issued 21 July 1989, Trauth et al., "Monoclonal Antibody-Mediated Tumor Regression by Induction of Apoptosis", pages 301-305.	16-19
Y, P	US 5,500,432 A (K.C. NICOLAOU et al.) 19 March 1996. See columns 10-12, claims 1-4.	20-26
X, P	Cell, Vol. 81, issued 19 May 1995, Stanger et al., "RIP: A Novel Protein containing a Death Domain that interacts with Fas/APO-1 (CD95) in yeast and causes Cell Death.	1-27

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/05386

A. CLASSIFICATION OF SUBJECT MATTER:  
IPC (6):

C12Q 1/68; G01N 33/35; C12P 21/04, 21/06; C12N 5/00; C07K 1/00, 16/00; A01N 43/04; C07H 21/04